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ORIGINAL ARTICLE

Gene cluster for biosynthesis of thermophilin 1277 – a lantibiotic produced by *Streptococcus thermophilus* SBT1277, and heterologous expression of TepI, a novel immunity peptide

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Abstract

Aims: To identify genes cluster for thermophilin 1277 produced by *Streptococcus thermophilus* SBT1277.

Methods and Results: To identify genes for thermophilin 1277 production, the chromosomal DNA region surrounding the structural gene, *tepA*, was sequenced using a primer-walking method. The thermophilin 1277 biosynthesis gene locus (*tep*) is a 9·9-kb region, which consists of at least ten open reading frames (ORFs) in the following order: *tepAMTFEGKRI* and *ORF4*. Homology analysis showed high similarity to genes involved in bovicin HJ50 production by *Streptococcus bovis* HJ50. *tepI* encodes a novel, small, positively charged hydrophobic peptide of 52 amino acids, which contains a putative transmembrane segment. By heterologous expression in *Lactococcus lactis* ssp. *cremoris* MG1363, the TepI-expressing strain exhibited at least 1·3 times higher resistance to thermophilin 1277.

Conclusions: Thermophilin 1277 biosynthesis genes were encoded by a 9·9-kbp region containing at least ten ORFs. TepI is a novel immunity peptide, which protected *Strep. thermophilus* SBT1277 against thermophilin 1277 in addition to TepFEG, a putative ABC transporter.

Significance and Impact of the Study: This is the first report regarding a lantibiotic gene cluster produced by *Strep. thermophilus* strain.

Introduction

Lantibiotics are synthesized as precursor peptides and subsequently undergo post-translational modifications (Kupke and Gotz 1996). Lanthionine and β-methyllantionine in these molecules are produced from the reaction of sulfhydryl groups of cysteine residues with dehydroalanine and dehydrobutyrine, after dehydration of serine and threonine residues, respectively. Jung (1991) divided the lantibiotics into two groups, type A (linear) and type B (globular), on the basis of their secondary structures. Sahl and Bierbaum (1998) further divided each group into three subgroups, the nisin (AI), lacticin 481 (AII) and lactococcin S (AIII) types, on the basis of the primary structural similarities of the peptides. Major lantibiotic gene clusters have been clarified, which include the structural gene (*lanA*), modification enzyme genes (*lan*B and *lanC*, or *lanM*), the secretion ABC-type transporter gene (*lanT*), the leader peptidase gene (*lanP*), self-protection (immunity) genes (*lanFEG* and *lanI*, or *lanH*) and regulatory genes (*lanR* and *lanK*). The gene cluster for nisin A is arranged in the sequence *nisABTCIPRKFEG* (McAuliffe *et al.* 2001).

Nisin A, an AI-type lantibiotic, is the most widely investigated, because it is the first found for bacteriocin and it is the only lantibiotic approved as a food preservative (Chatterjee *et al.* 2005). The successful application of

nisin as a safe food preservative has attracted much attention over the 50 countries (Delves-Broughton *et al.* 1996). So far, streptococcal lantibiotics have been reported in the oral cavities and upper respiratory tracts of humans and animals. However, they may also be isolated from almost any type of clinical specimen. Some examples include salivaricin A produced by *Streptococcus salivarius* 20P3 (Ross *et al.* 1993), mutacin 1140 produced by *Streptococcus mutans* JH1000 (Hillman *et al.* 1998) and streptococcin SA-M49 produced by *Streptococcus pyrogenes* (Hynes *et al.* 1994). Lantibiotic-producing oral streptococci is pathogenic; therefore, it is obvious that they cannot be used in foods. *Streptococcus macedonicus* ACA-DC 198, which was isolated from the cheese and nonpathogenic streptococcal species, produced a lantibiotic, macedocin (Papadelli *et al.* 2007).

There are numerous reports and reviews on LAB bacteriocins produced by lactococci, lactobacilli, pediococci, leuconstocs and enterococci; however, to date, only three class IIa bacteriocins produced by *Streptococcus thermophilus* strains have been cloned and sequenced – thermophilin 13 (produced by *Strep. thermophilus* Sfi 13) (Marciset *et al.* 1997), thermophilin A (produced by *Strep. thermophilus* ST134) (Ward and Somkuti 1995; Whitford *et al.* 2001) and thermophilin 9 (produced by *Strep. thermophilus* LMD-9) (Fontaine and Hols 2008). Furthermore, lantibiotics produced by *Strep. thermophilus* strains have never been reported. *Streptococcus thermophilus* is one of the most widely used bacteria in the manufacture of dairy products (yoghurt and cheese) and is considered to be a *generally recognized as safe* (GRAS) organism. Recently, the complete genome sequences of three *Strep. thermophilus* strains were published, and comparative genome analyses were performed (Hols *et al.* 2003; Bolotin *et al.* 2004). Comparative genomics and multilocus sequencing analyses suggest that this species is undergoing a process of regressive evolution, to adapt to growth in milk. While pathogenic streptococci are recognized for their high capacity to expose proteins at the cell surfaces to achieve cell adhesion or to escape host immune systems, *Strep. thermophilus* has lost these features, along with many virulence-related functions. These results are strongly suggestive of the safety status of *Strep. thermophilus* and the bacteriocins it produces.

Thermophilin 1277, produced by *Strep. thermophilus* SBT1277, is an AII-type lantibiotic, which contains two β-methyllanthionines and a disulfide bridge. Thermophilin 1277 is identical in terms of primary amino acid sequence to bovicin HJ50 from *Streptococcus bovis* HJ50 (Xiao *et al.* 2004). Furthermore, the structural gene *tepA* has been cloned (Kabuki *et al.* 2009).

Here, we sequenced the biosynthesis gene cluster of thermophilin 1277 and examined the heterologous expression of a small hydrophobic immunity peptide, TepI, which shows no similarity to other lantibiotic accessory proteins.

Materials and methods

Bacterial strains, plasmids and culture conditions

The strains and plasmids used in this study are listed in Table 1. The bacteriocin-producing strain *Strep. thermophilus* SBT1277 was isolated from raw milk (Kabuki *et al.* 2007). All strains were maintained as frozen stocks at −80°C. M17 medium (Becton, Dickinson & Co., Sparks, MD, USA) with 0·5% (w/v) glucose (GM17) was routinely used for culturing *Strep. thermophilus* SBT1277 at 37°C, and *Lactococcus lactis* ssp. *cremoris* MG1363 at 30°C. To select and maintain transformants, erythromycin (Sigma, Tokyo, Japan) was used at a concentration of 5 μg ml^{-1} in GM17 for *L. lactis* ssp. *cremoris* MG1363.

Molecular cloning and nucleotide sequencing

Molecular cloning techniques were performed as described by Sambrook *et al.* (1989). Chromosomal DNA was prepared using a previously reported method (Pospiech and Neumann 1995). PuReTaq Ready-To-Go PCR Beads (GE Health Bioscience, Tokyo, Japan) were used for the PCR, which was performed in a GeneAmp PCR System 9700 (PE Applied Biosystems, Tokyo, Japan) using standard PCR conditions. PCR products were purified using a QIAquick Spin Column (Qiagen, Tokyo, Japan). Plasmid extraction from *L. lactis* ssp. *cremoris* MG1363 was performed using a Plasmid Miniprep kit (Bio-Rad, Tokyo, Japan). DNA sequencing was performed by an industrial sequence commission (Hokkaido System Science, Sapporo, Japan). The nucleotide sequence surrounding *tepA* was determined by primer walking, using

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Description	Source or reference
Strains		
Streptococcus thermophilus SBT1277	Thermophilin 1277 producer	SBT
Lactococcus lactis ssp. *cremoris* MG1363	Plasmid-free derivative of NCDO712	Gasson (1983)
Plasmids		
pIL253-P32	Emr: pIL253 derivative with P32 promoter	Kemperman *et al.* (2003)
pTEPI	Emr: pIL253-P32 derivative carrying *tepI*	This study

Emr, erythromycin resistance; SBT, Snow Brand culture collection.

the total DNA of *Strep. thermophilus* SBT1277 as the template.

Computer analysis

Open reading frames (ORFs) were identified using an ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Sequence similarity searches of nucleotide or protein sequences were performed using the BLAST program in the DDBJ databases. Transmembrane regions in peptides and proteins were deduced using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosiu/) (Hirokawa *et al.* 1998). G+C content was calculated using a web tool (http://www.bioinformatics.org/JaMBW/3/1/9/index.html).

Cloning of recombinant carrying putative immunity peptide for thermophilin 1277

The *tepI* region was amplified using PuReTaq Ready-To-Go PCR Beads (GE Health Bioscience) with the primers 5′CGCGGATCCAAAGGCTCGAAAAATGGGTTA3′ and 5′GCAGGACGTCTCCCCAATCGCTAAAGCTAC3′ (the *Bam*HI and *Pst*I sites were underlined in the primer sequences, respectively), and the genomic DNA of *Strep. thermophilus* SBT1277 was used as a template. After digestion of the PCR products with *Bam*HI and *Pst*I, the *tepI* fragment was ligated into pIL253-P32 and digested with the same restriction enzymes. The ligation mixture was used to transform pIL253-P32 into *L. lactis* ssp. *cremoris* MG1363, and the resultant plasmid was named pTEPI.

Broth-based assay with recombinants of *Lactococcus lactis* ssp. *cremoris*

Bacteriocin tolerance was assayed using the agar well diffusion methods (Kabuki *et al.* 2007), which are briefly described later. The GM17 culture supernatant of *Strep. thermophilus* SBT1277 containing thermophilin 1277 was serially diluted by the 50 mmol l^{-1} sterile phosphate buffer (pH 6·8). The GM17 agar plate was overlaid with a soft agar lawn (15 ml), which was inoculated with a diluted overnight culture of each indicator strain at 1% (v/v). Wells, 6·88 mm in diameter, were cut from the plates, and 40 μl of serially diluted bacteriocin sample was added to each well. *Lactococcus lactis* ssp. *cremoris* MG1363, MG1363 (pIL253-P32) and MG1363 (pTEPI) were used as indicator strains. The diameters of the halos around wells of GM17 agar plates were measured after incubation at 30°C for 18 h. The experiments were performed in triplicate.

Nucleotide sequence accession number

The nucleotide and protein sequences of the thermophilin 1277 region have been assigned GenBank accession number AB434921.

Results

Cloning and sequencing of the gene cluster for thermophilin 1277 production

The nucleotide sequence of a 16 402-bp region flanking the structural gene of thermophilin 1277 (*tepA*) was determined using a primer-walking method. The G+C content (33%) of the total region was different from the average G+C content of *Strep. thermophilus* (39%) (Hols *et al.* 2003; Bolotin *et al.* 2004). Using computer analysis, 15 ORFs were detected; nine ORFs were designated *tepM*, *tepT*, *tepF*, *tepE*, *tepG*, *tepK*, *tepR*, *tepI* and *ORF4* in a 9·9-kb region containing *tepA*. The amino acid sequences of the seven *tep* gene (*tepAMTEGKR*) products among nine putative thermophilin 1277 biosynthesis genes show the highest similarity (98–100%) with those of the respective gene products of bovicin HJ50 produced by *Strep. bovis* HJ50 (Liu *et al.* 2009) (Fig. 1, Table 2).

TepM (837 amino acids), which is present downstream of *tepA*, showed 99% similarity to *bovM* (ACA51935) of *Strep. bovis* HJ50. The gene clusters of AII- and AIII-type lantibiotics contain a *lanM* (-like) gene encoding 900–1000 amino acid residues, which has been proposed to be involved in post-translational modification of lantibiotics such as lactosin S and lacticin 481. The function of LanM for AII- and AIII-type lantibiotic is dehydration of amino

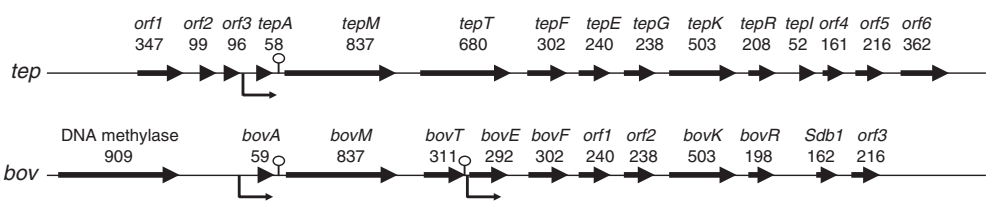

Figure 1 Comparison of the gene clusters for thermophilin 1277 and bovicin HJ50. The names of genes and a protein are shown with the amino acid length. Predicted promoters and a terminator are represented by angled arrows and an omega-like symbol, respectively.

Table 2 Deduced peptides and proteins derived from *tep*

ORF	Length (amino acids)	Proposed function	Best homologue [identities (%, no. of amino acids), GenBank accession no.	Localization (TMS no)*	pI	Molecular weight (kDa)
ORF1	347	Unknown	Helicase, *Streptococcus equi* ssp. *zooepidemicus* (95%, 2281 aa), YP002745281	Soluble (0)	5·30	41·0
ORF2	99	Unknown	Conserved domain protein, *Streptococcus suis* 89/1591 (88%, 99 aa), ZP03624205	Soluble (0)	4·15	12·0
ORF3	96	Unknown	Membrane protein, *Strep. equi* ssp. *zooepidemicus* (73%, 89 aa), YP002745279	Membrane (3)	6·07	10·9
tepA	58	Prethermophilin 1277	Bovicin HJ50, *Streptococcus bovis* HJ50 (100%, 58 aa), AAP23217	Soluble (0)	4·44	6·1
tepM	837	Post-translational modification	BovM, *Strep. bovis* HJ50 (99%, 837 aa), ACA51935	Soluble (0)	5·71	97·6
tepT	680	Processing and secretion ABC transporter.	BovT, *Strep. bovis* HJ50 (99%, 311 aa), ACA51936, BovE, *Strep. bovis* HJ50 (98%, 292 aa), ACA51937	Membrane (6)	8·38	77·3
tepF	302	Subunit of ABC transporter involved in immunity. ATP-binding protein	Lantibiotic transport ATP-binding protein, *Strep. suis* SC84 (63%, 304 aa), YP003024866	Soluble (0)	5·70	33·8
tepE	240	Subunit of ABC transporter involved in immunity. ATP transporter	Unknown, *Strep. bovis* HJ50 (98%, 240 aa), ACA51938	Membrane (7)	9·30	27·4
tepG	238	Subunit of ABC transporter involved in immunity. ATP transporter	Unknown, *Strep. bovis* HJ50 (98%, 238 aa), ACA51939	Membrane (6)	9·11	27·3
tepK	503	Histidine kinase	BovK, *Strep. bovis* HJ50 (99%, 503 aa), ACA51940	Membrane (8)	8·06	58·1
tepR	208	Response regulator	BovR, *Strep. bovis* HJ50 (98%, 198 aa), ACA51941	Soluble (0)	4·99	24·1
tepI	52	Immunity protein	Not hit	Membrane (2)	10·02	6·0
ORF4	161	Unknown	Sdb1, *Strep. bovis* HJ50 (100%, 161 aa), ACA51942	Membrane (1)	5·27	18·0
ORF5	216	Unknown	Unknown. *Strep. bovis* HJ50 (99%, 216 aa), ACA51943	Membrane (1)	5·44	24·4
ORF6	362	Unknown	DNA primase. *Strep. suis* BM407 (78%, 358 aa), NP735785	Soluble (0)	5·97	41·7

*TMS, transmembrane segment number, deduced using the SOSUI program.

acid and formed the thioether bridges in the molecule. On other hand, the function of Lan C for AI-type lantibiotic is to form the thioether bridges in the molecule. Uguen *et al.* (2000) identified 11 and eight conserved domains (N1–11 and C1–8) in the N- and C-terminal parts of LanM containing those from lacticin 481 groups, respectively. The LanM and LanC gene products shared some structural motifs, such as GXAHG, WCXG and CHG, in which histidine and cysteine residues are well conserved. These motifs might have some relevance in the catalytic function of these enzymes (Paul *et al.* 2007). The N-terminal region of TepM showed homology between six conserved domains of LanM proteins (positions 222–261, 274–284, 350–372, 385–398, 437–459 and 467–495). The C-terminal regions of TepM showed high similarity to the five domain motifs (positions 547–558, 661–673, 703–715, 752–758 and 787–816) including the consensus sequences of GXAHG (position 662), WCXG (position 706) and CHG (position 753) of LanM and lanC, which are predicted to form a thioether bridge-forming enzyme for lantibiotics (Li *et al.* 2006; Li and van der Donk 2007; Zhang *et al.* 2007). These findings suggest that TepM catalyses the reaction of prelantibiotic post-translation modification, which results in the formation of β-methyllanthionine residues.

TepT (680 aa) is deduced to possess an N-terminal proteolytic domain for cleavage of the leader sequence from

the prepeptide at the two conserved sequence motifs: QX4D/ECX2AX3MX3MX4Y/FGX4I/L and HY/FY/VV-X10I/LXDP (Havarstein *et al.* 1995) at positions 10 and 88, respectively. TepT also contains an ATP-binding domain, with Walker A motifs, GXXGXGKS/(position 498), and Walker B motif, *hhhh*DEP/A (*h*: a hydrophobic amino acids; positions 621) in a C-terminal domain (Fath and Kolter 1993). *tepT* has 99 and 98% similarity with *bovT* (ACA51936) and *bovE* (ACA51937) of *Strep. bovis* HJ50, respectively (Liu *et al.* 2009). These findings suggest that TepT has dual functions – the cleavage of the leader peptide (by the N-terminal domain) and the secretion of thermophilin 1277 as an ABC transporter (by the C-terminal domain).

The three ORFs, *tepF*, *tepE* and *tepG*, encode 302, 240 and 238 amino acids, respectively. TepF exhibits high similarity (39–63%) with proteins of immunity gene clusters for lacticin 481-type lantibiotics possessing the consensus ATP-binding domain with Walker motifs A and B at positions 36 and 156, respectively. TepE is predicted to be a membrane protein containing seven transmembrane segments. TepG was also a membrane protein containing six transmembrane segments. These results suggest that TepF, TepE and TepG are associated in an ABC transporter-like complex that functions in the immunity of *Strep. thermophilus* 1277 against thermophilin 1277.

tepKR encodes regulatory proteins for a typical two-component signal transduction system; TepK (503 aa) and TepR (208 aa) exhibited 99% similarity to BovK, a membrane-bound sensor histidine kinase, and BovR, a response regulator, of *Strep. bovis* HJ50.

TepI, a putative small peptide consisting of 52 amino acids, showed no similarity to any reported proteins encoded by lantibiotic gene clusters. The hydrophobic profile revealed that TepI is a highly cationic peptide with two putative membrane-spanning domains (Fig. 2). The ORF4 encoding 161 amino acid residues was identified as the disulfide oxidoreductase (Sdb1; ACA51942) by *Strep. bovis* HJ50 (Liu *et al.* 2009), which has a thioredoxin domain and exhibits 50% similarity to BlpGst from *Strep. thermophilus* LMD-9 (Gasson 1983) and BdbB from *Bacillus subtilis* 168 (Dorenbos *et al.* 1998; Pag *et al.* 1999) containing the conserved thioredoxin motif (CXXC). The formation of a disulfide bond in thermophilin 1277 might be related to the function of ORF4 (Sdb1). The ORF5 encoding 216 amino acid residues was 99% similarity to unknown protein from *Strep. bovis* HJ50. ORF5 and ORF6 (both downstream of ORF4), which had no similarity to protein by lantibiotic gene cluster reported, appeared to be unrelated to the biosynthesis of thermophilin 1277.

A putative promoter was located upstream of *tepA* (Kabuki *et al.* 2009). A deduced terminator was predicted to be present downstream of *tepA*. However, deduced terminator sequences were not found downstream of other regions related to bacteriocin synthesis. The G+C content of the area between ORF1 and ORF3 and that between *tepA* and *orf4* was 39 and 29%, respectively. ORF4, which is downstream of the putative *tep* operon, contained 33% G+C. On the other hand, the downstream region of ORF4 exhibited 37% G+C content.

Heterogonous expression of the putative immunity peptide for thermophilin 1277

In silico analysis indicated that TepI – a novel hydrophobic peptide with the cationic region – is the immunity-like peptide for thermophilin 1277. Figure 2 shows the primary sequence and deduced transmembrane regions of the small immunity peptides for the lantibiotics. The peptides, TepI (for thermophilin 1277), EciI (for epicidin 280), PepI (for Pep5), LtnI (lacticin 3147) and NukI (nukacin ISK-1), ranging in size from 52 to 116 amino acid residues, have two or three transmembrane segments.

Figure 2 Primary sequences for (putative) immunity peptides of LAB bacteriocins. TepI, EciI, PepI, LtnI and NukH are immunity peptides for thermophilin 1277, epicidin 280, Pep5, lacticin 3147 and nukcin ISK-I, respectively. The transmembrane regions deduced using SOSUI program are underlined. The residue positions and lengths of the region are shown within parentheses.

tepI was cloned downstream of the lactococcal P32 promoter in pIL253-P32, and heterologous expression of *tepI* from the resulting plasmid, pTEPI (Fig. 3), was examined in *L. lactis* ssp. *cremoris* MG1363. The recombinant strain, *L. lactis* ssp. *cremoris* MG1363 (pTEPI), was at least 1·3 times more resistant to the GM17 culture supernatant of *Strep. thermophilus* SBT1277 than the control strain carrying pIL253-P32 (Fig. 4).

Discussion

By sequencing the gene cluster containing the structural gene (*tepA*) for a lantibiotic, thermophilin 1277, a modification enzyme gene (*tepM*), an ABC transporter gene (*tepT*), self-protection genes (*tepFEGI*) and two-component regulation genes (*tepRK*) were deduced. These ORFs (except for *tepI*) are commonly found in the biosynthetic gene clusters of lacticin 481-type lantibiotics (Dufour *et al.* 2006).

Thermophilin 1277 is identical to bovicin HJ50 of *Strep. bovis* HJ50 in the structural genes. The biosynthetic gene cluster of bovicin HJ50 (*c.* 9·9 kbp) (Liu *et al.* 2009) contains at least 10 ORFs (*bovA*, *M*, *T*, *E*, *F*, *ORF1*, *ORF2*, *K*, *R* and *ORF3*) involved in biosynthesis, regulation and immunity, which correspond to each gene of *tepAMTFEGRK-ORF4*, except for *tepI*. However, the putative *tepT* product consisted of 680 aa, instead of 311 aa from the putative *bovT* product. This shows that

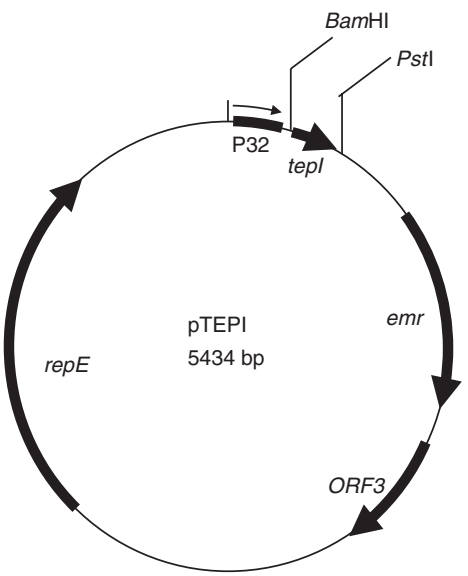

Figure 3 Plasmid map of the *tepI* expression vector pTEPI. P32, the lactococcal constitutive promoter; *tepI*, the putative thermophilin 1277 immunity gene; *emr*, the erythromycin resistance gene; *ORF3*, an uncharacterized ORF in the vector pIL253; and *repE*, the replication protein E gene.

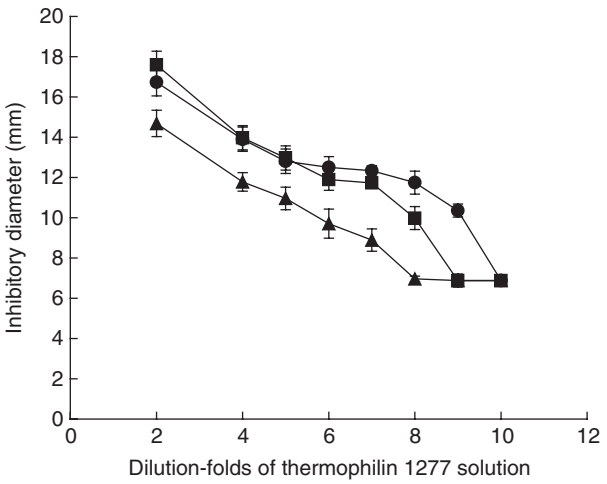

Figure 4 Immunity assay for the recombinants of *Lactococcus lactis* ssp. *cremoris* MG1363 against thermophilin 1277. The immunity levels of *L. lactis* ssp. *cremoris* MG1363 (●), MG1363 (pIL253-P32) (■) and MG1363 (pTEPI) (▲) were investigated by agar-well diffusion assay using serially diluted culture supernatants of *Streptococcus thermophilus* SBT1277 (thermophilin 1277 solution).

TepT has both a proteolytic domain in its N-terminal and an ATP-binding domain in the C-terminal corresponding to BovT that has a proteolytic domain and BovE (292 aa) that has an ATP-binding domain, respectively. Based on the molecular size and estimated function difference between BovT and TepT, we conclude TepT may possess the activities of both BovT and BovE. *TepF*, *E* and *G* are the putative immunity genes corresponding to the deduced genes of bovicin HJ50, *bovF*, *ORF1* and *ORF2*, respectively. *TepI* product has no similarity to other bacteriocin biosynthesis protein. As the homology analysis of *orf5 and orf6* products show no similarity to the bacteriocin biosyntheses protein, these protein might be not involved with thermophilin 1277 expression.

Two immunity (self-protecting) systems for lantibiotics have been reported. The first consists of individual immunity proteins (generically termed LanI or LanH) that are located in the cytoplasmic membrane or at the surface and prevent interaction of bacteriocin(s) with the membrane. The other consists of the ABC transporters (usually composed of two or three subunits, generically termed (LanFE(G)), which actively export intracellularly accumulated bacteriocins to the outside (e.g., NisFEG). LanI (LanH) and LanFE(G) may act alone, or in combination, which results in cooperative functionality (Draper *et al.* 2008)

A LanI immunity system has been detected in some major lantibiotics, such as nisin (NisI, 245 resides), subtilin (SpaI, 165 residues), Pep5 (PepI, 69 residues), lacticin 3147 (LtnI, 116 residues), epicidin 280 (EciI, 62 residues)

and cytolycin (CylI, 327 residues) (Kuipers *et al.* 1993; Klein and Entian 1994; Qiao *et al.* 1995; Paik *et al.* 1998; McAuliffe *et al.* 2000; Stein *et al.* 2003, 2005; Hoffmann *et al.* 2004; Reis *et al.* 2004). NisI and SpaI are lipoproteins that are anchored in the cytoplasmic membrane by their lipid motifs; they capture lantibiotics to avoid bacteriocin action. LtnI contains a leucine zipper motif and three hydrophobic domains, which suggests that it is a transmembrane protein that forms homodimers (McAuliffe *et al.* 2000).

LanFEG is the only immunity system for the lacticin 481 families, such as lacticin 481 (Piard *et al.* 1992), mutacin II (Woodruff *et al.* 1998) and streptococcin A-FF22 (McLaughlin *et al.* 1999). Aso *et al.* (2005) reported a novel type of lantibiotic-binding peptide, NukH, which contributes to the immunity of nukacin ISK-1. NukH (92 aa) has three putative membrane-spanning domains (Fig. 4) and is similar to the LanI peptide for lacticin 3147. Together, the two immunity systems of NukH and NukFEG provide a much greater degree of immunity function than each system alone. NukH has been referred to as an accessory factor, which acts as an ancillary protein for the assembly of the functioning ABC transporter.

We demonstrated the heterologous expression of *tepI* with the P32 promoter in *L. lactis* ssp. *cremoris* MG1363. The recombinant strain is not completely resistant to thermophilin 1277, but is at least 1·3 times more resistant to thermophilin 1277 than the control strains carrying pIL253-P32. This result shows that the *tepI* product is an immunity peptide against thermophilin 1277 and might cooperate with the *tepFEG* products for full immunity function. This suggests that the immunity system of thermophilin 1277 slightly differs from that of bovicin HJ50.

The ORF4 downstream of *tepI* encodes a putative disulfide oxidase-like protein (161 aa). BlpGst of *Strep. thermophilus* LMD-9 (Fontaine and Hols 2008) and BdbB of *B. subtilis* 168 (Dorenbos *et al.* 1998) have been reported to be disulfide oxidases involved in bacteriocin production. Bovicin HJ50, which is identical to thermophilin 1277 with regard to the primary amino acid structure, has a thiol-disulfide oxidoreductase gene in the bacteriocin loci (Liu *et al.* 2009). Gene disruption analysis showed that the thiol-disulfide oxidoreductase encoded by *sdb1* is not involved in bovicin HJ50 biosynthesis. The inhibitory activity of the reduced form of bovicin HJ50 treated by dithiothreitol (DTT) neither decreased nor increased (Xiao *et al.* 2004). On the other hand, our previous result shows that the antimicrobial activity of thermophilin 1277 disappears after treatment with DTT. It can be speculated that ORF4 functions in the formation of intra- and/or intermolecular disulfide bridges between the cysteine residues of thermophilin 1277. The function of *ORF4* products is being investigated.

The G+C content of *tepAMTFEGRKI-ORF4* (29%) is lower than the average genome G+C content (*c.* 38%) of *Strep. thermophilus* (Hols *et al.* 2003; Bolotin *et al.* 2004). Horizontal gene transfer (HGT) can be defined as the exchange of genetic material between phylogenetically unrelated organisms (Jain *et al.* 2002). Mechanisms of intercellular DNA transfer include conjugation, phage transduction and transformation. HGT events can be detected using phylogenetic and compositional approaches. Information on the gene transfer mechanisms for genes, such as transposase- or phage-related genes found in the neighbourhood of the target genes, can improve the prediction of HGT events; there are many reports regarding the HGT events indicated. The operon of lacticin 481, which is 23% similar to thermophilin 1277, contains part of Tn*5721* – a potentially mobile DNA sequence in a 70-kbp plasmid (Dufour *et al.* 2006). In contrast, no plasmids were detected in the strains of *Streptococcus pyogenes* and *Streptococcus agalactiae* producing streptococcin A-F22 and salivaricin A1 (45 and 42% similarity to thermophilin 1277, respectively). This indicates that the lantibiotic loci are chromosomal in these species (Wescombe *et al.* 2006). It should be noted that a transposase gene lies downstream of the streptococcin A-F22 gene cluster in *Strep. pyogenes* (McLaughlin *et al.* 1999), which might explain plasmid–chromosome and/or chromosome–plasmid exchanges. The other gene clusters of the lacticin 481 group are chromosomally located. Transposase genes have been found in the mutacin II cluster (Cheng *et al.* 1999), as well as between the *macA* and *macR* genes for macedocin (Papadelli *et al.* 2007). Consistently, the G+C content of the macediocin gene cluster (29·6%) is low compared to the average genome G+C content of *Strep. macedonicus* species (38%) (Papadelli *et al.* 2007). The surrounding region of the macedocin gene cluster is involved in the three putative mobilization elements: relaxase, transposase and resolvase; this suggests that *Strep. macedonicus* might be able to gain the macedocin genes by horizontal transfer of DNA from other organisms. These findings might indicate the possibility of horizontal transfer for the thermophilin 1277 gene cluster in *Strep. thermophilus* SBT1277.

Streptococcus thermophilus is of major importance to the food industry; it is the second most important industrial dairy starter after *L. lactis* (Hols *et al.* 2003). *Streptococcus thermophilus* SBT1277 is a food-grade GRAS microorganism, which is isolated from milk and is routinely used as a starter organism by various sectors of the food industry. The safety status of *Strep. thermophilus* SBT1277 will be of considerable advantage in the application of this bacteriocin-producing organism as a biopreservative.

References

Aso, Y., Okuda, K., Nagao, J., Kanemasa, Y., Phuong, N.T.B., Koga, H., Shioya, K., Sashihara, T. *et al.* (2005) A novel type of immunity protein, NukH, for the lantibiotic nukacin ISK-I produced by *Staphylococcus warnneri* ISK-1. *Biosci Biotechnol Biochem* **69**, 1403–1410.

Bolotin, A., Quinquis, B., Renault, P., Sorokin, A., Ehrlich, S.D., Kulakauskas, S., Lapidus, A., Goltsman, E. *et al.* (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nat Biotechnol* **27**, 1554–1558.

Chatterjee, C., Paul, M., Xie, L. and van der Donk, W.A. (2005) Biosynthesis and mode of action of Lantibiotics. *Chem Rev* **105**, 633–683.

Cheng, P., Qi, F., Novak, J. and Caufield, P.W. (1999) The specific genes for lantibiotic mutacin II biosynthesis in *Streptococcus mutans* T8 are clustered and can be transferred en bloc. *Appl Environ Microbiol* **65**, 1356–1360.

Delves-Broughton, J., Blackburn, P., Evans, R.J. and Hugenholtz, J. (1996) Applications of bacteriocin, nisin. *Antonie Van Leeuwenhoek* **69**, 193–202.

Dorenbos, R., Stein, T., Kabel, J., Bruand, C., Bolhuis, A., Bron, S., Quax, W.J. and Van Dijl, J.M. (1998) Thiol-disulfide oxidoreductases are essential for the production of the lantibiotic sublancin 168. *J Biol Chem* **273**, 16682–16688.

Draper, L.A., Ross, R.P., Hill, C. and Cotter, P.D. (2008) Lantibiotic immunity. *Curr Protein Pept Sci* **9**, 39–49.

Dufour, A., Hindre, T., Haras, D. and LePennec, J.P. (2006) The biology of lantibiotic from the lacticin 481 group is coming of age. *FEMS Microbiol Rev* **31**, 134–167.

Fath, M.J. and Kolter, R. (1993) ABC transporter: bacterial exporters. *Microbiol Rev* **57**, 995–1017.

Fontaine, L. and Hols, P. (2008) The inhibitory spectrum of Thermophilin 9 from *Streptococcus thermophilus* LMD-9 depends on the production of multiple peptides and the activity of BlpGst, a thiol-disulfide oxidase. *Appl Environ Microbiol* **74**, 1102–1110.

Gasson, M.J. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol* **154**, 1–9.

Havarstein, L.S., Diep, D.B. and Nes, I.F. (1995) A family of bacteriocin ABC transportes carry out proteolytic processing of their substrates concomitant with export. *Mol Microbiol* **16**, 229–240.

Hillman, J.D., Novak, D.J., Sagura, E., Guitierrez, J.A., Brooks, T.A., Crowley, P.J., Hess, M., Azizi, A. *et al.* (1998) Genetic and biochemical analysis of mutacin 1140, a lantibiotic from *Streptococcus mutans*. *Infect Immun* **66**, 2743–2749.

Hirokawa, T., Boon-Chieng, S. and Mitaku, S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**, 378–379.

Hoffmann, A., Schnieder, T., Pag, U. and Sahy, H.G. (2004) Localization and functional analysis of PepI, the immunity peptide of Pep5 producing *Staphylococcus epidermidis* strain 5. *Appl Environ Microbiol* **70**, 3263–3271.

Hols, P., Hancy, F., Fontaine, L., Grossiord, B., Prozzi, D., Leblond-Bourget, N., Decaris, B., Bolotin, A. *et al.* (2003) New insights in the molecular biology and physiology of *Streptococcus thermophilus* revealed by comparative genomics. *FEMS Microbiol Rev* **29**, 435–463.

Hynes, W.L., Friendand, V.L. and Ferretti, J.J. (1994) Duplication of the lantibiotic structural gene in M-type 49 group A streptococcus strains producing streptococcin A-M49. *Appl Environ Microbiol* **60**, 4207–4209.

Jain, R., Rivera, M.C., Moore, M.C. and Lake, J.A. (2002) Horizontal gene transfer in microbial genome evolution. *Theor Popul Biol* **61**, 489–495.

Jung, G. (1991) Lantibiotics: a survey. In *Nisin and Novel Lantibiotic* ed. Jung, G. and Sahl, H.-G. pp. 1–34. Leiden, The Netherlands: Escom Publishers.

Kabuki, T., Uenishi, H., Watanabe, M., Seto, Y. and Nakajima, H. (2007) Characterization of a bacteriocin, thermophilin 1277, produced by *Streptococcus thermophilus* SBT1277. *J Appl Microbiol* **102**, 971–980.

Kabuki, T., Uenishi, H., Seto, Y., Yoshioka, T. and Nakajima, H. (2009) A unique lantibiotic, thermophilin 1277, containing a disulfide bridge and two thioether bridges. *J Appl Microbiol* **106**, 853–862.

Kemperman, R., Jonker, M., Nauta, A., Kuipers, O.P. and Kok, J. (2003) Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. *Appl Environ Microbiol* **69**, 5839–5848.

Klein, C. and Entian, K.D. (1994) Genes involved in self-protection against the lantibiotic subtilin produced by *Bacillus subtilis* ATCC 663. *Appl Environ Microbiol* **60**, 2793–2801.

Kuipers, O.P., Beerthuyqen, M.M., Siezen, R.J. and de Vos, W.M. (1993) Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*. Requirement of expression of the nisA and nisI genes for development of immunity. *Eur J Biochem* **216**, 281–291.

Kupke, T. and Gotz, F. (1996) Post-translational modifications of lantibiotics. *Antonie Van Leeuwenhoek* **69**, 139–150.

Li, B. and van der Donk, W.A. (2007) Identification of essential catalytic residues of the cyclase NisC involved in the biosynthesis of nisin. *J Biol Chem* **282**, 21169–21175.

Li, B., Yu, J.S., Brunzelle, J.S., Moll, G.N., van der Donk, W.A. and Nair, S.K. (2006) Structure and mechanism of the lantibiotic cyclase involved in nisin biosynthesis. *Science* **311**, 1464–1467.

Liu, G., Zhong, J., Ni, J., Chen, M., Xiao, H. and Huan, L. (2009) Characteristics of the bovicin HJ50 gene cluster in *Streptococcus bovis* HJ50. *Microbiology* **155**, 584–593.

Marciset, O., Jeronimus-Stratingh, M.C., Mollet, B. and Poolman, B. (1997) Thermophilin 13, a nontypical

antilisterial poration complex bacteriocin that functions without a receptor. *J Biol Chem* **272**, 14277–14284.

McAuliffe, O., Hill, C. and Ross, R.P. (2000) Identification and overexpression of ltnI, a novel gene which confers immunity to the two-component lantibiotic lacticin 3147. *Microbiology* **146**, 129–138.

McAuliffe, O., Ross, R.P. and Hill, C. (2001) Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol Rev* **25**, 285–308.

McLaughlin, R.E., Ferretti, J.J. and Hynes, W.L. (1999) Nucleotide sequence of the streptococcin A-FF22 lantibiotic regulon: model for production of the lantibiotic SA-FF22 by strains of model for production of the lantibiotic SA-F22 by strains of *Streptococcus pyrogenes*. *FEMS Microbiol Lett* **175**, 171–177.

Pag, U., Heidrich, C., Bierbaum, G. and Sahl, H.G. (1999) Molecular analysis of expression of the lantibiotic Pep5 immunity phenotype. *Appl Environ Microbiol* **65**, 591–598.

Paik, S.H., Chakicherla, A. and Hansen, J.N. (1998) Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *J Biol Chem* **273**, 23134–23142.

Papadelli, M., Karsioti, A., Anastasiou, R., Georgalaki, M. and Tsakalidou, E. (2007) Characterization of the gene cluster involved in the biosynthesis of macedocin, the lantibiotic produced by *Streptococcus macedonicus*. *FEMS Microbiol Lett* **272**, 75–82.

Paul, M., Patton, G.C. and van der Donk, W.A. (2007) Mutants of the zinc ligands of lacticin 481 synthetase retain dehydration activity but have impaired cyclization activity. *Biochemistry* **46**, 6268–6276.

Piard, J.C., Kuipers, O.P., Rollema, H.S., Desmazeaud, M.J. and de Vos, W.M. (1992) Structure, organization, and expression of the lct gene for lacticin 481, a novel lantibiotic produced by *Lactococcus lactis*. *J Biol Chem* **58**, 16361–16368.

Pospiech, A. and Neumann, B. (1995) A versatile quick-prep of genomic DNA from gram-positive bacteria. *Trends Genet* **4**, 217–218.

Qiao, M., Immonen, T., Koponen, O. and Saris, P.E.J. (1995) The cellular location and effect on nisn immunity of the NisI protein from *Lactococcus lactis* N8 expressed nisI *Escherichia coli* and *L. lactis*. *FEMS Microbiol Lett* **131**, 75–80.

Reis, M., Eschbach-Bludau, M., Iglesias-Wind, M.I., Kupke, T. and Sahl, H.G. (2004) Producer immunity towards the lantibiotic Pep5: identification of the immunity gene *pepI* and localization and functional analysis of its gene product. *Appl Environ Microbiol* **60**, 2876–2883.

Ross, K.F., Ronson, C.W. and Tagg, J.R. (1993) Isolation and characterization of the lantibiotic salivaricin A and its structural gene *salA* from *Streptococcus salivarius* 20P3. *Appl Environ Microbiol* **59**, 2014–2021.

Sahl, H.G. and Bierbaum, G. (1998) Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from gram-positive bacteria. *Annu Rev Microbiol* **30**, 41–79.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press.

Stein, T., Heinzmann, S., Solovieva, I. and Entian, K.D. (2003) Function of *Lactobacillus lactis* nisin immunity gene nisI and nis FEG after coordinated expression in the surrogate host *Bacillus subtilis*. *J Biol Chem* **278**, 89–94.

Stein, T., Heinzmann, S., Dusterhus, S., Borchert, S. and Entian, K.D. (2005) Expression and functional analysis of the subtilin immunity gene spaIFEG in the subtilin-sensitive hosat *Bacillus subtilis* MO1099. *J Bacteriol* **187**, 822–828.

Uguen, P., Le Pennec, J.P. and Dufour, A. (2000) Lantibiotic biosynthesis: interaction between prelacticin 481 and its putative modification enzyme, LctM. *Appl Environ Microbiol* **182**, 5262–5266.

Ward, D.J. and Somkuti, G.A. (1995) Characterization of a bacteriocin produced by *Streptococcus thermophilus* ST134. *Appl Microbiol Biotechnol* **43**, 330–335.

Wescombe, P.A., Burton, J.P., Cadieux, P.A., Klesse, N.A., Hyink, O., Heng, N.C.K., Chilocott, C.N., Reid, G. *et al.* (2006) Megaplasmids encode differing combinations of lantibiotics in *Streptococcus salivarius*. *Antonie Van Leeuwenhoek* **90**, 269–280.

Whitford, M.F., McPherson, M.A., Forster, R.J. and Teather, R.M. (2001) Identification of bacteriocin-like inhibitors from rumen *Streptococcus* spp. and isolation and characterization of bovicin 255. *Appl Environ Microbiol* **67**, 569–574.

Woodruff, W.A., Novak, J. and Caufield, P.W. (1998) Sequence analysis of *mutA* and *mutM* genes involved in the biosynthesis of the lantibiotic mutacin II in *Streptococcus mutans*. *Gene* **206**, 37–43.

Xiao, H., Chen, X., Chen, M., Tang, S., Zhao, X. and Huan, L. (2004) Bovicin HJ50, a novel lantibiotic produced by *Streptococcus bovis* HJ50. *Microbiology* **150**, 103–108.

Zhang, X., Ni, W. and van der Donk, W.A. (2007) On the regioselectivity of thioether formation by lacticin 481 synthetase. *Org Lett* **9**, 3343–3346.